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Production of an antibody Fab fragment using 2A peptide in insect cells

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[**Key words:** Insect cell; 2A peptide; GSG linker; Furin cleavage site; Fab fragment; High Five cell; Recombinant protein production]

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Abstract

Antibody Fab fragments consist of heavy chain (Hc) and light chain (Lc) polypeptides assembled with a disulphide bond. The production of a recombinant Fab fragment requires the simultaneous expression of two genes encoding both an Hc and an Lc in the same host cell. In the present study, we investigated the production of Fab fragments in lepidopteran insect cells using a bicistronic plasmid vector carrying the Hc and Lc genes linked with a 2A self-cleaving peptide sequence from the porcine teschovirus-1. We also examined the arrangement of a GSG spacer sequence and a furin cleavage site sequence with the 2A sequence. Western blot analysis and enzyme-linked immunosorbent assay (ELISA) of culture supernatants showed that *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells transfected with a plasmid in which the Hc and Lc genes were joined by the 2A sequence successfully secreted Fab fragments with antigen-binding activity after self-cleavage of the 2A peptide. The GSG linker enhanced 2A cleavage efficiency, and the furin recognition site was useful for removal of 2A residues from the Hc. Transfection with a single plasmid that contained sequences for GSG, the furin cleavage site, GSG, and the 2A peptide between the Hc and Lc genes exhibited a higher productivity than co-transfection with a set of plasmids separately carrying the Hc or Lc gene. These results demonstrate that bicistronic expression with the appropriate combination of a furin recognition site, GSG linkers, and a 2A peptide may be an effective way to efficiently produce recombinant antibody molecules in insect cells.

Introduction

Monoclonal antibodies and antibody fragments including Fab fragments have been widely used for therapeutics and diagnostics, because they bind to antigens with high levels of both affinity and specificity. Currently, mammalian cells such as Chinese hamster ovary (CHO) cells and NS0 cells have been generally used as host cells for the stable expression of recombinant therapeutic antibodies (1, 2). Insect cells have recently been recognized as an excellent platform for the production of biologically active recombinant proteins including antibody molecules (3–5). Insect cells are easier and more cost-effective to cultivate than mammalian cells, and they can be grown to a high cell density in suspension with a serum-free medium. They can also produce significant amounts of recombinant proteins through post-translational processing and modifications that are similar to those performed in mammalian cells (6, 7).

Among antibody molecules, an IgG consists of two identical heavy chains (Hc) and two identical light chains (Lc), which assemble with disulphide bonds, and an Fab fragment consists of two chains comprised of an Fd fragment (Hc) and an Lc, joined by a disulphide bond. Hence, the production of an IgG or an Fab fragment requires the simultaneous expression of two genes encoding both Hc and Lc in the same cell.

Bicistronic expression vectors have been developed to co-express two genes from a single open reading frame driven by one promoter. In bicistronic expression systems, internal ribosome entry site (IRES) sequences, which are retained in the picornavirus family, have been widely used. An IRES allows the initiation of translation in a cap-independent manner, i.e., ribosomes bind internally at the initiating AUG without scanning the 5' non-translated region of the transcript (8), thereby ensuring the co-expression of genes located before and after the IRES. However, the use of an IRES

sequence has several limitations (9, 10). Since the size of an IRES sequence is usually longer than 500 nucleotides, limited cloning capacity could be a problem when a large insert and/or a large plasmid must be used along with an IRES sequence. Furthermore, the translation efficiency of genes separated by an IRES element is not equivalent, because the translation efficiency of a gene placed after an IRES is much lower than that of a gene located before an IRES (11).

2A peptides were also identified among the picornavirus family but in different sub-groups such as in Aphthovirus and in equine rhinitis A virus (9). 2A peptides are relatively short oligopeptides (approximately 20 amino acids) located between the viral P1 and P2 proteins. The 2A peptides can undergo self-cleavage between the last two amino acids by preventing the formation of a normal peptide bond between glycine and the last proline in order to generate mature P1 and P2 proteins, which results in the ribosome skipping to the next codon during protein translation (9, 10, 12). Similar 2A sequences have been found in several other viruses; the most commonly used are from the foot-and-mouth disease virus (F2A) (13), the equine rhinitis A virus (E2A), the *Thosea asigna* virus (T2A), and the porcine teschovirus-1 (P2A). Reportedly, P2A has a higher cleavage efficiency than F2A, E2A, or T2A in human cell lines, zebrafish, mice, and *Drosophila* (10, 14). Often, 2A peptides are used in mammalian cells such as CHO cells (15–17) and human embryonic kidney 293 cells (12) for IgG production. In CHO cells and mice, the use of a 2A self-processing sequence resulted in a higher level of protein productivity compared with that gained by the use of an IRES (12, 17). Whereas some papers have reported recombinant protein production using 2A peptides in insect cells, such as Sf9 cells (18), cells derived from *Bombyx mori* (18, 19), and *Drosophila* S2 cells (14), there have been no reports on antibody production utilizing a 2A peptide in insect cells.

In the present study, we constructed bicistronic plasmid vectors in which the Hc and Lc genes of an Fab fragment were linked with a P2A self-cleavage peptide sequence, and we investigated the production of Fab fragments in lepidopteran insect cells transfected with a single plasmid. The use of a glycine-serine-glycine (GSG) linker and a furin cleavage site between an Hc and a 2A peptide was also examined. A GSG linker motif is known to improve the self-cleavage efficiency of the 2A peptide (18, 20) by affording greater flexibility (21). Furin is a ubiquitous endonuclease localized in the Golgi apparatus and is highly conserved among eukaryotic species (22, 23). A furin cleavage site is a sequence that is recognized by the proteinase, and it has been added between an Hc and a 2A peptide to remove the 2A residue that remains upstream of the C-terminus of the 2A (12, 16, 24). Furthermore, we also referred to the arrangements of a 2A peptide, a GSG linker, a furin cleavage site, an Hc, and an Lc for efficient production of Fab fragments in insect cells.

MATERIALS AND METHODS

Insect cells and the culture medium *Trichoplusia ni* BTI-TN-5B1-4 (High Five; Thermo Fisher Scientific, Waltham, MA, USA) cells were cultured in a serum-free medium Express Five SFM (Thermo Fisher Scientific) supplemented with 2.6 g/L L-glutamine (Nacalai Tesque, Kyoto, Japan) and 10 mg/L gentamycin sulfate (Thermo Fisher Scientific). The cells were maintained at 27°C in T-flasks in a non-humidified incubator (25). Cell density was determined by microscopically counting the number of cells using the Countess II automated cell counter (Thermo Fisher Scientific), while cell viability was judged by the exclusion of trypan blue dye.

Construction of 2A-mediated bicistronic vectors For the expression of an Fab fragment from a single open reading frame driven by one promoter, the Hc and Lc genes of the 3A21 mouse anti-RNase A (26) Fab fragment were joined by the DNA sequence coding for the 2A peptide from the porcine teschovirus-1 (18). The resultant DNA fragments were cloned into the plasmid vector pIHAneo (3), which contained the *B. mori* nucleopolyhedrovirus (BmNPV) IE-1 transactivator, the BmNPV HR3 enhancer, and the *B. mori* actin promoter for high-level expression in insect cells (3, 27). The *Drosophila* immunoglobulin heavy chain binding protein (BiP) signal sequence (3) was employed upstream of the Hc and Lc genes, respectively (Fig. 1). DNA fragments encoding a GSG linker and a furin recognition site were inserted upstream of the 2A peptide sequence to improve 2A cleavage efficiency and to remove residues of the 2A peptide left after 2A cleavage, respectively, as shown in Fig. 1. The amino acid sequences of the 2A peptide, the GSG linker, and the furin recognition site are shown in Table 1. All the DNA sequences were codon-optimized for lepidopteran insect cells.

← Fig. 1

← Table 1

Transient expression and cell culture Transfection grade linear polyethylenimine hydrochloride (PEI) (PEI MAX; Mw 40,000; Polysciences, Warrington, PA, USA) was used as a transfection agent. PEI was prepared at a concentration of 1 g/L in 150 mM NaCl, pH 7.0 and sterilized by filtration through a 0.22 μm membrane filter.

In a static culture, High Five cells in the exponential growth phase were inoculated with fresh medium into 6-well plates at a density of 2×10^5 cells/cm^3 an hour before transfection. For each transfection, 1 μg of plasmid DNA and 2 μg of PEI per 10^5 cells were mixed in 150 mM NaCl and incubated at room temperature for 5 min. After incubation, the complex of DNA and PEI was added to the cells, and the cells were then

statically incubated at 27°C. As a positive control, High Five cells were co-transfected with a total of 1 μg of pIHAneo separately containing the Hc or Lc gene (5) at a ratio (w/w) of 1:1 (Fig. 1 a). After 72 h, culture supernatants were separated from the cell suspensions via centrifugation and analyzed for the production of Fab fragments. To determine transfection efficiency, the plasmid pXINSECT-EGFP carrying the enhanced green fluorescent protein (EGFP) gene was co-transfected as previously described (5). At 72 h after co-transfection, the cell suspension was removed, and the numbers of green fluorescent cells and total cells were determined using a flow cytometer (Guava easyCyte 5HT, Merck Millipore, Darmstadt, Germany).

In a shake flask culture, cells in the exponential growth phase were suspended at a density of 2×10^5 cells/cm^3 in fresh medium. Fifteen ml of the cell suspension was transferred into a 100-ml screw-capped Erlenmeyer flask. For each transfection, 0.5 μg of plasmid DNA and 1 μg of PEI per 10^5 cells were mixed in 150 mM NaCl and incubated at room temperature for 5 min. The DNA and PEI complex was added to the cells, and the cells were then incubated at 27°C on a rotary shaker (90 rpm). As a positive control, High Five cells were co-transfected with a total of 0.5 μg of pIHAneo individually carrying either the Hc or Lc gene at a ratio (w/w) of 1:1 (Fig. 1 a). At the appropriate time, 500 μl of the cell suspension was sampled to measure the cell density, and the culture supernatants were analyzed for the production of Fab fragments.

Western blot analysis Culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% gel under reduced and non-reduced conditions. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using the iBlot 2 dry blotting system (Thermo Fisher Scientific). Immunoreactive proteins were detected using alkaline

phosphatase-conjugated anti-mouse IgG (H + L) (Promega, Madison, WI, USA), which is polyclonal antibody that binds to both Hc and Lc of mouse IgG, and staining was initiated using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium (Promega).

Enzyme-linked immunosorbent assay (ELISA) Culture supernatants were also analyzed by ELISA to evaluate the concentration of Fab fragments with antigen-binding activity as previously described (5). In brief, ELISA plates were coated with bovine RNaseA as the antigen, and horseradish peroxidase-conjugated anti-mouse IgG (H + L) (Promega) was used. The ELISA POD substrate TMB kit (Nacalai Tesque) was used for detection according to the manufacturer's protocol. The absorbance was measured with a microplate reader (EnSpire; PerkinElmer Japan, Yokohama, Japan) using a test wavelength of 450 nm and a reference wavelength of 650 nm. The difference between the two absorbances was converted to an Fab fragment concentration by interpolating the value on a predetermined standard curve. The anti-RNaseA Fab fragments were purified from the culture supernatant with NHS-activated Sepharose 4 (GE Healthcare, Little Chalfont, UK) coupled with bovine RNaseA, and were used as a standard.

Statistical analysis Statistically significant differences among data sets were determined using Student's *t*-test. A p-value < 0.05 was considered as statistically significant.

RESULTS

Production of Fab fragments using 2A peptide To examine 2A peptide-

mediated antibody production in insect cells, we constructed a bicistronic expression vector carrying the Hc and Lc genes of the 3A21 Fab fragment linked with a 2A self-cleaving peptide sequence from the porcine teschovirus-1 (18). High Five cells were either transfected with a single bicistronic vector (Fig. 1 b) or were co-transfected with a set of plasmid vectors individually containing either the Hc or Lc gene (Fig. 1 a) in 6-well plates. At 72 h after transfection, culture supernatants were collected, and the production of Fab fragments was investigated by western blotting under both non-reduced and reduced conditions. Under non-reduced conditions (Fig. 2A), specific protein bands ← Fig. 2 were detected at electrophoretic mobilities of approximately 50 and 25 kDa in the culture supernatant of the cells transfected with the bicistronic vector (Hc-2A-Lc) as well as the cells co-transfected with the set of plasmid vectors (Hc & Lc). The electrophoretic mobility coincided with the molecular weight of the 3A21 Fab fragment and that of the Lc monomer, respectively. It should be noted that High Five cells as well as mammalian cells do not secrete Hc alone (5). Under reduced conditions (Fig. 2B), specific protein bands corresponding to Hc and Lc monomers were obtained at approximately 25 kDa in the culture supernatant of the cells transfected with the bicistronic vector (Hc-2A-Lc) and of the cells co-transfected with the Hc and Lc genes (Hc & Lc). These results indicate that High Five cells transfected with the bicistronic vector using the 2A sequence successfully secreted Fab fragments in the culture supernatant after self-cleavage of the 2A peptide occurred in the cells. However, a low-intensity protein band corresponding to uncleaved Hc-2A-Lc was observed at approximately 50 kDa in the culture supernatant of the cells transfected with the bicistronic vector (Fig. 2B).

The effects of a GSG linker and a furin cleavage site were then investigated. When a GSG linker was added to the N-terminus of the 2A peptide (Fig. 1 c), the approximately 50 kDa uncleaved protein band disappeared on the western blots under reduced conditions

(Fig. 2B, Hc-GSG-2A-Lc), demonstrating that the GSG linker enhanced the 2A cleavage efficiency. When a furin cleavage site was added to the N-terminus of the 2A peptide (Fig. 1 d), under non-reduced conditions the approximately 50 kDa band corresponding to the Fab fragment shifted to a smaller molecular weight (Fig. 2A; Hc-furin-2A-Lc), which indicates that cleavage successfully occurred at the furin recognition site, and then the residual 2A peptide was removed from the Hc. Hence, the furin cleavage site is useful for the production of Fab fragments without 2A residue in insect cells.

The effects of the 2A peptide, the GSG linker, and the furin cleavage site on Fab fragment production were also examined by ELISA of the culture supernatants. The production of Fab fragments with antigen-binding activity was enhanced by adding the GSG linker to the N-terminus of the 2A peptide (Hc-GSG-2A-Lc) as compared with Hc-2A-Lc (Fig. 3; $p = 0.032$). Fab fragment production was depressed by adding the furin cleavage site alone upstream of the 2A peptide (Hc-furin-2A-Lc) compared with Hc-2A-Lc. However, the production was improved by using the furin cleavage site along with the GSG linker (Fig. 3; Hc-furin-GSG-2A-Lc vs. Hc-furin-2A-Lc ($p = 0.012$); Hc-furin-GSG-2A-Lc vs. Hc-GSG-2A-Lc ($p = 0.058$)). The rank order of the GSG linker and the furin cleavage site at the N-terminus of the 2A peptide was important, because the plasmid vector Hc-furin-GSG-2A-Lc resulted in productivity that was higher than that with the vector Hc-GSG-furin-2A-Lc (Fig. 3; $p = 0.0087$). Finally, transfection with the bicistronic expression vector that contained sequences for GSG, the furin cleavage site, GSG, and the 2A peptide between the Hc and Lc genes (Hc-GSG-furin-GSG-2A-Lc) exhibited the highest productivity at a roughly two-fold higher level than conventional co-transfection with a set of plasmids separately containing either the Hc or Lc gene (Hc & Lc) (Fig. 3; $p = 0.00084$). The transfection efficiency was determined as the percentage of EGFP-positive cells to total cells by flow cytometry at 72 h after

← Fig. 3

transfection as previously described (5). The transfection efficiency with the bicistronic vector Hc-GSG-furin-GSG-2A-Lc was almost the same as that obtained by co-transfection of the Hc and Lc genes (data not shown).

Effect of arranging the Lc gene as the first cistron Translation efficiency of the first cistron placed before an IRES was much higher than that of the second cistron located after the IRES (11, 16). Ho et al. (16) showed that arrangement of the Lc gene as the first cistron also returned a higher level of IgG expression compared with use of the Hc gene as the first cistron in 2A-mediated vectors. Higher Lc/Hc gene ratios led to higher levels of Fab fragment secretion in co-transfection of the Lc and Hc genes (5, 16). Taken together, higher productivity of Fab fragments might be expected when the Lc is arranged at the N-terminus of the 2A peptide (Fig. 1 h, i). In the present study, when the plasmid vectors Lc-GSG-2A-Hc and Lc-furin-GSG-2A-Hc were transfected with High Five cells, lower amounts of Fab fragments were detected in both western blotting and ELISA than from the plasmids Hc-GSG-2A-Lc and Hc-furin-GSG-2A-Lc, respectively (Figs. 2C and 3). Interestingly, in western blot analysis under reduced conditions (Fig. 2D), approximately 50 kDa uncleaved protein bands were slightly detected.

Cleavage at the furin recognition site without 2A To examine the cleavage efficiency of Hc and Lc at the furin recognition site without 2A peptide, the plasmid vectors Hc-furin-Lc and Hc-furin-GSG-Lc (Fig. 1 j, k) were transfected with High Five cells for Fab fragment production. In western blot analysis under non-reduced conditions (Fig. 2E), protein bands corresponding to the Fab fragment were obtained with the plasmid vectors Hc-furin-Lc and Hc-furin-GSG-Lc as well as with Hc-furin-GSG-2A-Lc, but an Lc monomer was hardly visible at approximately 25 kDa with both Hc-

furin-Lc and Hc-furin-GSG-Lc. Under reduced conditions (Fig. 2F), only uncleaved protein bands were detected at approximately 50 kDa and neither Lc nor Hc monomers were observed with Hc-furin-Lc and Hc-furin-GSG-Lc plasmid vectors. Furthermore, in ELISA (Fig. 3), Fab fragments with antigen-binding activity were scarcely detectable with Hc-furin-Lc and Hc-furin-GSG-Lc. These results confirm that the furin cleavage site alone is insufficient to cleave Hc and Lc and produce Fab fragments with antigen-binding activity.

Production of Fab fragments in a shake-flask culture Since the plasmid vectors Hc-furin-GSG-2A-Lc and Hc-GSG-furin-GSG-2A-Lc gave the highest productivity of Fab fragments with antigen-binding activity in a static culture (Fig. 3), these vectors were individually transfected into High Five cells for Fab fragment production in a shake-flask culture. Cells transfected with bicistronic expression vectors grew in a manner similar to the cells co-transfected with the Hc and Lc genes (Fig. 4A). ← Fig. 4 However, transfection with Hc-furin-GSG-2A-Lc and Hc-GSG-furin-GSG-2A-Lc plasmid vectors exhibited a somewhat higher concentration of Fab fragments than co-transfection (Hc & Lc) (Fig. 4B). In the shake culture, the difference in the productivity of Fab fragments was not as significant as in the static culture. The reason for this is not clear, but the difference in the productivity in a shake-flask culture might increase when transfection conditions are optimized. Nevertheless, the plasmid Hc-GSG-furin-GSG-2A-Lc gave the highest productivity of Fab fragments in both static and shake-flask cultures.

DISCUSSION

Production of recombinant antibody molecules such as an Fab fragment as well as an IgG requires a simultaneous expression of two genes encoding both Hc and Lc in an identical cell. In the present study, we constructed plasmid vectors using a 2A self-cleaving peptide sequence from the porcine teschovirus-1 with various combinations of GSG linkers and a furin recognition site for bicistronic expression of the Hc and Lc genes of an Fab fragment (Fig. 1). Western blot analysis (Fig. 2) and ELISA (Fig. 3) of culture supernatants showed that High Five cells transfected with the Hc and Lc genes linked with the 2A peptide sequence successfully secreted Fab fragments with antigen-binding activity following self-cleavage of the 2A peptide. The concentration of Fab fragments with antigen-binding activity secreted from the cells transfected with the plasmid vector using the 2A sequence alone (Hc-2A-Lc) was lower than that from the cells co-transfected with the Hc and Lc genes (Hc & Lc), but Fab fragment production was elevated by means of adding a GSG linker and a furin cleavage site at the N-terminus of the 2A peptide (Fig. 3).

The GSG linker enhanced 2A cleavage efficiency, because in western blot analysis under reduced conditions (Fig. 2B) approximately 50 kDa uncleaved protein bands disappeared by adding GSG to the N-terminus of the 2A peptide when Hc-GSG-2A-Lc and Hc-furin-GSG-2A-Lc were compared with Hc-2A-Lc and Hc-furin-2A-Lc, respectively. In addition, the production of Fab fragments with antigen-binding activity was dramatically enhanced approximately 2-fold by using the GSG spacer at the N-terminus of the 2A peptide, when comparing Hc-GSG-2A-Lc, Hc-furin-GSG-2A-Lc, and Hc-GSG-furin-GSG-2A-Lc with Hc-2A-Lc (p = 0.032), Hc-furin-2A-Lc (p = 0.0012), and Hc-GSG-furin-2A-Lc (p = 0.0024), respectively (Fig. 3). Reportedly, the mechanism of a GSG linker could involve reduction in the inhibition rate of the 2A reaction via the tertiary structure of the C-terminal region of the first protein upstream of

the 2A peptide (18, 21, 28).

The furin recognition site was useful for the removal of the 2A residues from the Hc because a comparison of Hc-furin-2A-Lc with Hc-2A-Lc in western blotting under non-reduced conditions (Fig. 2A) revealed that the approximately 50 kDa band corresponding to the Fab fragment was shifted to a smaller molecular weight by adding the furin cleavage site to the N-terminus of the 2A peptide. However, the arrangement of the furin cleavage site directly in front of the 2A sequence (Hc-furin-2A-Lc, Hc-GSG-furin-2A-Lc) produced a lower yield of Fab fragments than the use of the GSG sequence between the furin cleavage site and the 2A sequence (Hc-furin-GSG-2A-Lc, Hc-GSG-furin-GSG-2A-Lc), respectively (Fig. 3). Interestingly, under reduced conditions a specific band of approximately 28 kDa, probably corresponding to a furin-2A-Lc fusion protein, was detected with both Hc-furin-2A-Lc and Hc-GSG-furin-2A-Lc plasmid vectors. Reportedly, a GSG linker arranged between a furin recognition site and a 2A peptide could enhance the cleavage efficiency at both the furin and 2A cleavage sites by creating more favorable conformations via increased exposure of the furin recognition site at the protein surface and by preventing the inhibition of 2A cleavage caused by interference from the first protein upstream of the 2A peptide (18, 21). These previous studies have shown that a furin cleavage site should keep a proper distance from a 2A sequence in order to achieve efficient cleavage. Chng et al. also reported that only 2A from the porcine teschovirus-1 gave a remarkable difference in Fab fragment expression levels between the absence and presence of GSG between the furin cleavage site and the N-terminus of the 2A in CHO cells (21). Hence, when other 2A peptides (F2A, E2A, or T2A) were adopted in High Five cells, Fab fragment productivity might not be influenced by GSG linkers.

Reportedly, the order of Hc and Lc genes around a 2A sequence affected the 2A

cleavage efficiency and IgG productivity in CHO cells (16). Ho et al. showed that the arrangement of the Lc gene as the first cistron gave a higher level of IgG productivity than that of the Hc gene as the first cistron in both IRES- and 2A-mediated expressions in CHO cells (16). By contrast, in the present study, the arrangement of the Hc gene as the first cistron returned a higher level of Fab productivity (Figs. 2C, D and 3). It is unclear how the arrangement of either the Hc or Lc gene as the first cistron caused differences in the production of Fab fragments, but this might show a correlation with the differences between Hc genes in IgG and Fab fragments.

Linking the Hc and Lc genes with only the furin cleavage site without a 2A sequence produced mainly uncleaved Hc-furin-Lc or Hc-furin-GSG-Lc fusion proteins (Fig. 2F), and thus the production of Fab fragments with antigen-binding activity was hardly detectable (Fig. 3). These results agree with a recent study by Lin et al., which showed that linking Hc and Lc genes by a furin cleavage site alone resulted mainly in the production of Hc-furin-Lc fusion protein and a low amount of IgG in CHO cells (29). These results show that the furin cleavage site alone is insufficient to cleave Hc and Lc expressed from a single open reading frame.

In the present study, GSG-furin-GSG-2A-mediated expression of the Hc and Lc genes (Hc-GSG-furin-GSG-2A-Lc) exhibited the highest productivity of Fab fragments via either a static (Fig. 3) or a shake-flask culture (Fig. 4B) of transfected High Five cells. These results demonstrate that bicistronic expression using the appropriate arrangement of GSG linkers, a furin recognition site, and a 2A peptide allows the efficient production of antibody molecules consisting of multiple polypeptides in insect cells. The productivity in a shake-flask culture could be further improved by optimization of transfection conditions. More detailed analyses of Hc and Lc polypeptides after 2A cleavage, such as amino acid sequence determination, have not yet been performed. A

comparative study using different 2A peptides is also necessary in order to select the optimal 2A peptide in insect cells.

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References

1. **Butler, M. and Meneses-Acosta, A.:** Recent advances in technology supporting biopharmaceutical production from mammalian cells, Appl. Microbiol. Biotechnol., **96**, 885–894 (2012).
2. **Kunert, R. and Reinhart, D.:** Advances in recombinant antibody manufacturing, Appl. Microbiol. Biotechnol., **100**, 3451–3461 (2016).
3. **Yamaji, H., Manabe, T., Watakabe, K., Muraoka, M., Fujii, I., and Fukuda, H.:** Production of functional antibody Fab fragment by recombinant insect cells, Biochem. Eng. J., **41**, 203–209 (2008).
4. **Yamaji, H.:** Production of antibody in insect cells, pp. 53–76, in Al-Rubeai, M. (Ed.), Antibody expression and production. Cell engineering, vol. 7. Springer Science + Business Media, Dordrecht, The Netherlands (2011).
5. **Mori, K., Hamada, H., Ogawa, T., Ohmuro-Matsuyama, Y., Katsuda, T., and Yamaji, H.:** Efficient production of antibody Fab fragment by transient gene

expression in insect cells, J. Biosci. Bioeng., **124**, 221–226 (2017).

6. **Luckow, V. A.:** Protein production and processing from baculovirus expression vectors, pp. 51–90, in: Shuler, M. L., Wood, H. A., Granados, R. R., and Hammer, D. A. (Eds.), Baculovirus expression systems and biopesticides. Wiley-Liss, New York, USA (1995).
7. **Yamaji, H.:** Suitability and perspectives on using recombinant insect cells for the production of virus-like particles, Appl. Microbiol. Biotechnol., **98**, 1963–1970 (2014).
8. **Mountford, P. S. and Smith, A. G.:** Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis, Trends Genet., **11**, 179–184 (1995).
9. **Trichas, G., Begbie, J., and Srinivas, S.:** Use of the viral 2A peptide for bicistronic expression in transgenic mice, BMC Biol., 6, 40 (2008).
10. **Kim, J. H., Lee, S. R., Li, L. H., Park, H. J., Park, J. H., Lee, K. Y., Kim, M. K., Shin, B. A., and Choi, S. Y.:** High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice, PLoS One, **6**, e18556 (2011).
11. **Mizuguchi, H., Xu, Z., Ishii-Watabe, A., Uchida, E., and Hayakawa, T.:** IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector, Mol. Ther., 1, 376–382 (2000).
12. **Fang, J., Qian, J.-J., Yi, S, Harding, T. C., Tu, G. H., van Roey, M., and Jooss, K.:** Stable antibody expression at therapeutic levels using the 2A peptide, Nat. Biotechnol., **23**, 584–590 (2005).
13. **Robertson, B. H., Grubman, M. J., Weddell, G. N., Moore, D. M., Welsh, J. D., Fischer, T., Dowbenko, D. J., Yansura, D. G., Small, B., and Kleid, D. G.:** Nucleotide and amino acid sequence coding for polypeptides of foot-and-mouth

disease virus type A12, J. Virol., **54**, 651–660 (1985).

14. **Daniels, R.W., Rossano, A. J., Macleod, G. T., and Ganetzky, B.:** Expression of multiple transgenes from a single construct using viral 2A peptides in *Drosophila*. PLoS One, **9**, e100637 (2014).

15. **Davies, S. L., O'Callaghan, P. M., McLeod, J., Pybus, L. P., Sung, Y. H., Rance, J., Wilkinson, S. J., Racher, A. J., Young, R. J., and James, D. C.:** Impact of gene vector design on the control of recombinant monoclonal antibody production by Chinese hamster ovary cells, Biotechnol. Prog., **27**, 1689–1699 (2011).

16. **Ho, S. C., Bardor, M., Li, B., Lee, J. J., Song, Z., Tong, Y. W., Goh, L. T., and Yang, Y.:** Comparison of internal ribosome entry site (IRES) and Furin-2A (F2A) for monoclonal antibody expression level and quality in CHO cells, PLoS One, **8**, e63247 (2013).

17. **Ebadat, S., Ahmadi, S., Ahmadi, M., Nematpour, F., Barkhordari, F., Mahdian, R., Davami, F., and Mahboudi, F.:** Evaluating the efficiency of CHEF and CMV promoter with IRES and Furin/2A linker sequences for monoclonal antibody expression in CHO cells, PLoS One, **12**, e0185967 (2017).

18. **Wang, Y., Wang, F., Wang, R., Zhao, P, and Xia, Q.**: 2A self-cleaving peptide-based multi-gene expression system in the silkworm *Bombyx mori*, Sci. Rep., **5**, 16273 (2015).

19. **Wang, Y., Wang, F., Xu, S., Wang, R., Chen, W., Hou, K., Tian, C., Wang, F., Zhao, P., and Xia, Q:** Optimization of a 2A self-cleaving peptide-based multigene expression system for efficient expression of upstream and downstream genes in silkworm, Mol. Genet. Genomics, **294**, 849–859 (2019).

20. **Minskaia, E. and Ryan, M. D.:** Protein coexpression using FMDV 2A: effect of "linker" residues, Biomed. Res. Int., **2013**, 291730 (2013).

21. **Chng, J., Wang, T., Nian, R., Lau, A., Hoi, K. M., Ho, S. C., Gagnon, P., Bi, X., and Yang, Y.:** Cleavage efficient 2A peptides for high level monoclonal antibody expression in CHO cells, mAbs, **7**, 403–412 (2015).
22. **Gäken, J., Jiang, J., Daniel, K., van Berkel, E., Hughes, C., Kuiper, M., Darling, D., Tavassoli, M., Galea-Lauri, J., Ford, K., Kemeny, M., Russel,l S., and Farzaneh, F.:** Fusagene vectors: a novel strategy for the expression of multiple genes from a single cistron, Gene Ther, **7**, 1979–1985 (2000).
23. **de Felipe, P., Luke, G. A., Brown, J. D., and Ryan, M. D.:** Inhibition of 2A-mediated 'cleavage' of certain artificial polyproteins bearing N-terminal signal sequences, Biotechnol. J., **5**, 213–223 (2010).
24. **Fang, J., Yi, S., Simmons, A., Tu, G. H., Nguyen, M., Harding, T. C., van Roey, M., and Jooss, K.:** An antibody delivery system for regulated expression of therapeutic levels of monoclonal antibodies *in vivo*. Mol. Ther., **15**, 1153–1159 (2007).
25. **Furuta, T., Ogawa, T., and Yamaji, H.:** Production of antibody fragments using the baculovirus-insect cell system, Methods Mol. Biol., **907**, 371–387 (2012).
26. **Katakura, Y., Kobayashi, E., Kurokawa, Y., Omasa, T., Fujiyama, K., and Suga, K.-I.:** Cloning of cDNA and characterization of anti-RNase A monoclonal antibody 3A21, J. Ferment. Bioeng., **82**, 312–314 (1996).
27. **Douris, V., Swevers, L., Labropoulou, V., Andronopoulou, E., Georgoussi, Z., and Iatrou, K.:** Stably transformed insect cell lines: tools for expression of secreted and membrane-anchored proteins and high-throughput screening platforms for drug and insecticide discovery, Adv. Virus Res., **68**, 113–156 (2006).
28. **Hofacre, A., Yagiz, K., Mendoza, D., Lopez Espinoza, F., Munday, A. W., Burrascano, C., Singer, O., Gruber, H. E., Jolly, D. J., and Lin, A. H.:** Efficient

therapeutic protein expression using retroviral replicating vector with 2A peptide in cancer models, Hum. Gene Ther., **29**, 437–451 (2018).

29. **Lin, J., Neo, S. H., Ho, S. C. L., Yeo, J. H. M., Wang, T., Zhang, W., Bi, X., Chao, S.-H., and Yang, Y.:** Impact of signal peptides on furin-2A mediated monoclonal antibody secretion in CHO cells, Biotechnol. J., **12**, 1700268 (2017).

Figure captions

FIG. 1. Schematic representation of bicistronic vectors for Fab fragment production in insect cells. Each fragment was ligated with pIHAneo vector. *Drosophila* BiP signal sequence (striped box) was used upstream of every heavy chain (Hc) and light chain (Lc) gene of the 3A21 Fab fragment. As a positive control, the Hc or Lc gene of the Fab fragment was respectively ligated to pIHAneo (a), and High Five cells were co-transfected with both plasmid vectors. Only construct (a) contained both a V5 epitope tag sequence and a His tag sequence at the 3' end of the Lc gene (dotted box). Actin, *Bombyx mori* cytoplasmic actin promoter; 2A, DNA encoding a 2A peptide derived from the porcine teschovirus-1; G, DNA encoding a GSG linker; F, DNA encoding a furin recognition site; pA, OpIE2 polyadenylation sequence from the *Orgyia pseudotsugata* nucelopolyhedrovirus.

FIG. 2. Western blot analysis of culture supernatant of transfected cells under non-reduced (A, C, E) and reduced conditions (B, D, F). (A, B) Bicistronic vectors containing the Hc gene upstream of the 2A sequence and the Lc gene downstream of the 2A sequence (Fig. 1 a–g) were transfected with High Five cells in 6-well plates. Control, untransfected cells. (C, D) Bicistronic vectors containing the Lc gene as the first cistron in front of the Hc gene (Fig. 1 h, i) were transfected. (E, F) Bicistronic vectors without the 2A sequence (Fig. 1 j, k) were used.

FIG. 3. Enzyme-linked immunosorbent assay (ELISA) of the cell-culture supernatant. Bars represent the means with error bars indicating S.E. obtained from three independent experiments in static cultures. Control, untransfected cells. Asterisk indicates

1 significant difference (* $p < 0.05$; ** $p < 0.01$).

2

3 FIG. 4. Time course of viable cell density (A) and concentration of Fab fragments in
4 the culture supernatant (B) in shake-flask cultures of transfected High Five cells. Bars
5 represent the means with error bars indicating S.E. obtained from three independent
6 experiments.

7

8

TABLE 1. List of amino acid sequences

Name	Sequence
P2A	ATNFSLLKQAGDVEENPGP
Furin cleavage site	RRKR
GSG linker	GSG
Hc	MDVQLQESGPGLVKPSQSLSLTCTVTGYSITSDYAWNWI RQFPGNKLEWMGYISHSGSTGYNPSLKSRISITRDTSKN QFFLQLNSVTTEDTATYYCARGGKNWDAYWGQGTLVT VSSKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPV TVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPS ETVTCNVAHPASSTKVDKKIPRDCGAAALE
Lc	MDIKMTQSPSSMYAFLGERVTITCKASQDINSYLSWFQQ KPGKSPKTLIYRANRLVDGVPSRFSGSGSGQDYSLTISSL EYEDMGIYYCLQYDELPFTFGSGTKLEIKRADAAPTVSI FPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQ NGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTC EATHKTSTSPIVKSFNRNECAAALE
BiP signal	MKLCILLAVVAFVGLSLG

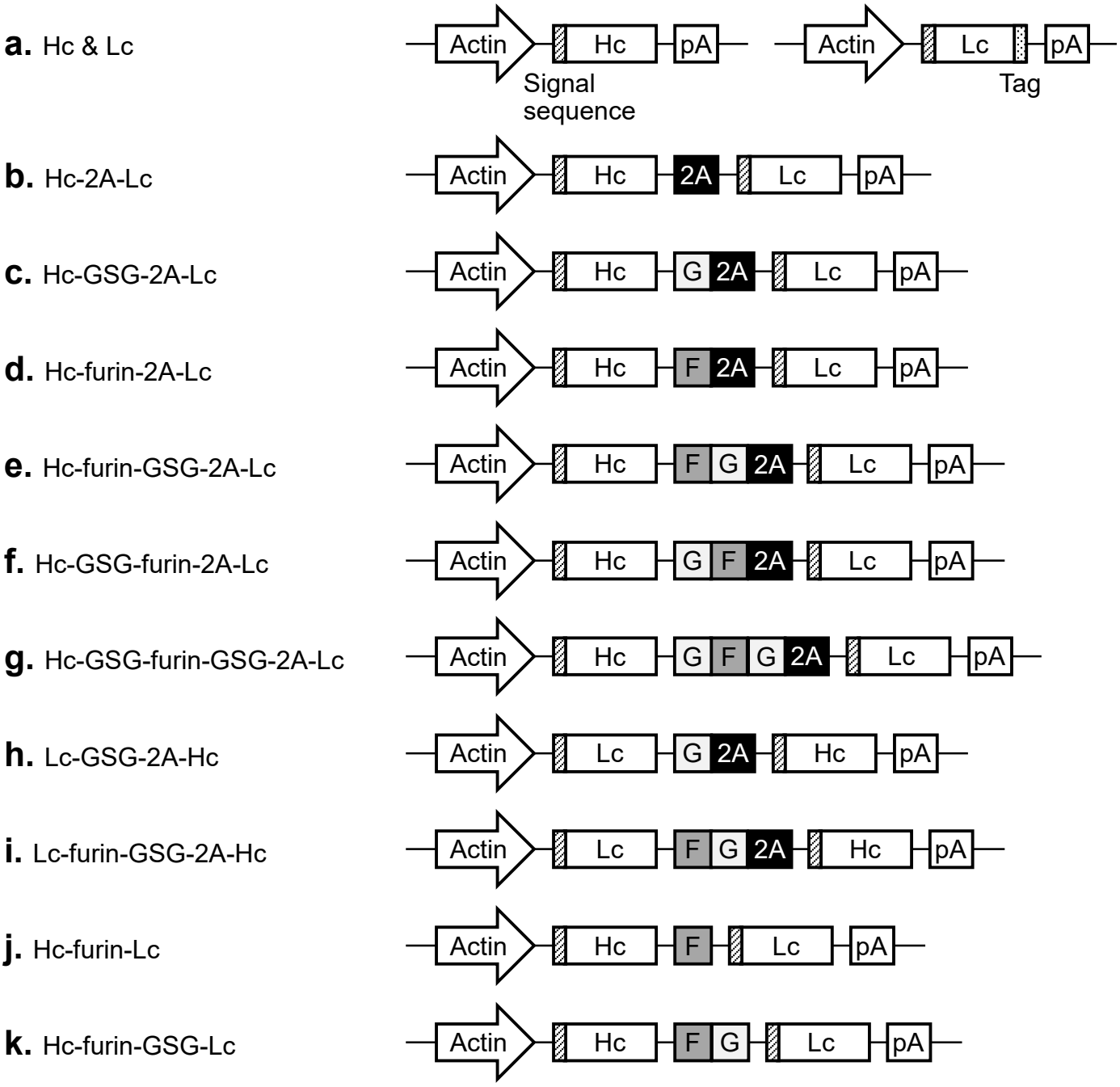
a. Hc & Lc
Actin
Hc
pA
Actin
Lc
pA
Signal sequence
Tag
b. Hc-2A-Lc
Actin
Hc
2A
Lc
pA
c. Hc-GSG-2A-Lc
Actin
Hc
G
2A
Lc
pA
d. Hc-furin-2A-Lc
Actin
Hc
F
2A
Lc
pA
e. Hc-furin-GSG-2A-Lc
Actin
Hc
F
G
2A
Lc
pA
f. Hc-GSG-furin-2A-Lc
Actin
Hc
G
F
2A
Lc
pA
g. Hc-GSG-furin-GSG-2A-Lc
Actin
Hc
G
F
G
2A
Lc
pA
h. Lc-GSG-2A-Hc
Actin
Lc
G
2A
Hc
pA
i. Lc-furin-GSG-2A-Hc
Actin
Lc
F
G
2A
Hc
pA
j. Hc-furin-Lc
Actin
Hc
F
Lc
pA
k. Hc-furin-GSG-Lc
Actin
Hc
F
G
Lc
pA

Fig. 1

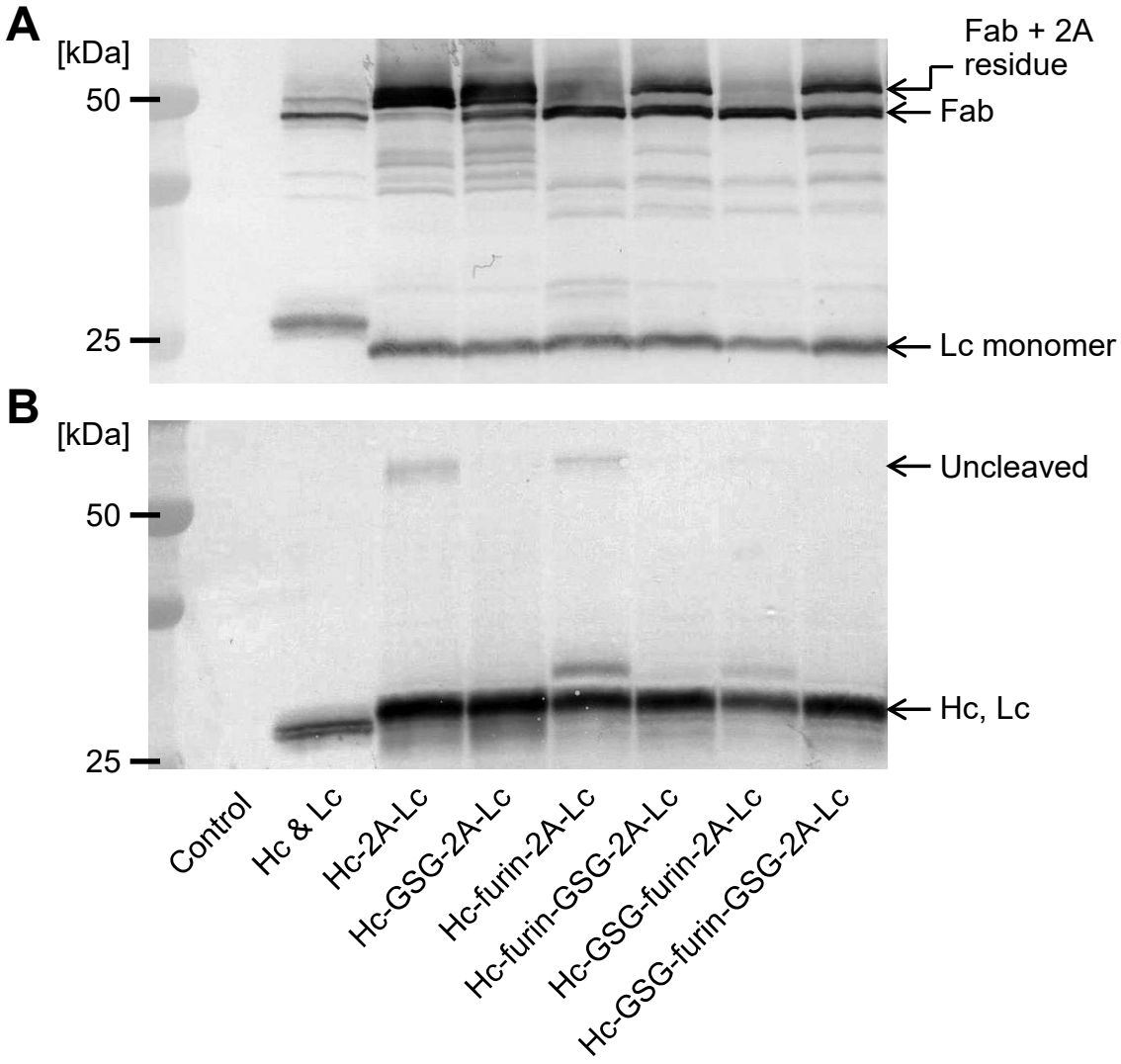
A
[kDa]
50
25
Fab + 2A residue
Fab
Lc monomer
B
[kDa]
50
25
Uncleaved
Hc, Lc
Control
Hc & Lc
Hc-2A-Lc
Hc-GSG-2A-Lc
Hc-furin-2A-Lc
Hc-furin-GSG-2A-Lc
Hc-GSG-furin-2A-Lc
Hc-GSG-furin-GSG-2A-Lc

Fig. 2 A, B

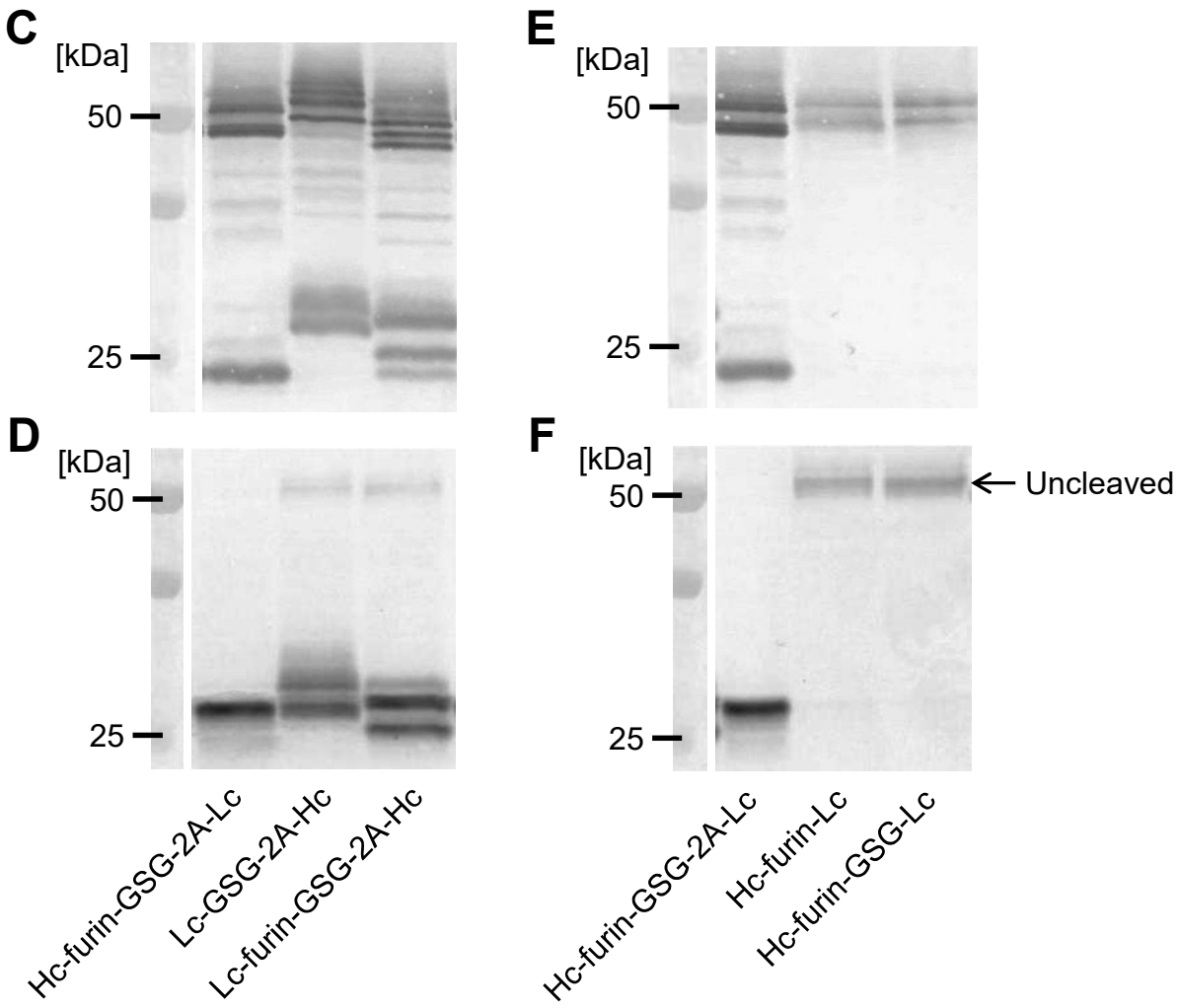
C
[kDa]
50
25
E
[kDa]
50
25
D
[kDa]
50
25
F
[kDa]
50
25
← Uncleaved
Hc-furin-GSG-2A-Lc
Lc-GSG-2A-Hc
Lc-furin-GSG-2A-Hc
Hc-furin-GSG-2A-Lc
Hc-furin-Lc
Hc-furin-GSG-Lc

Fig. 2 C–F

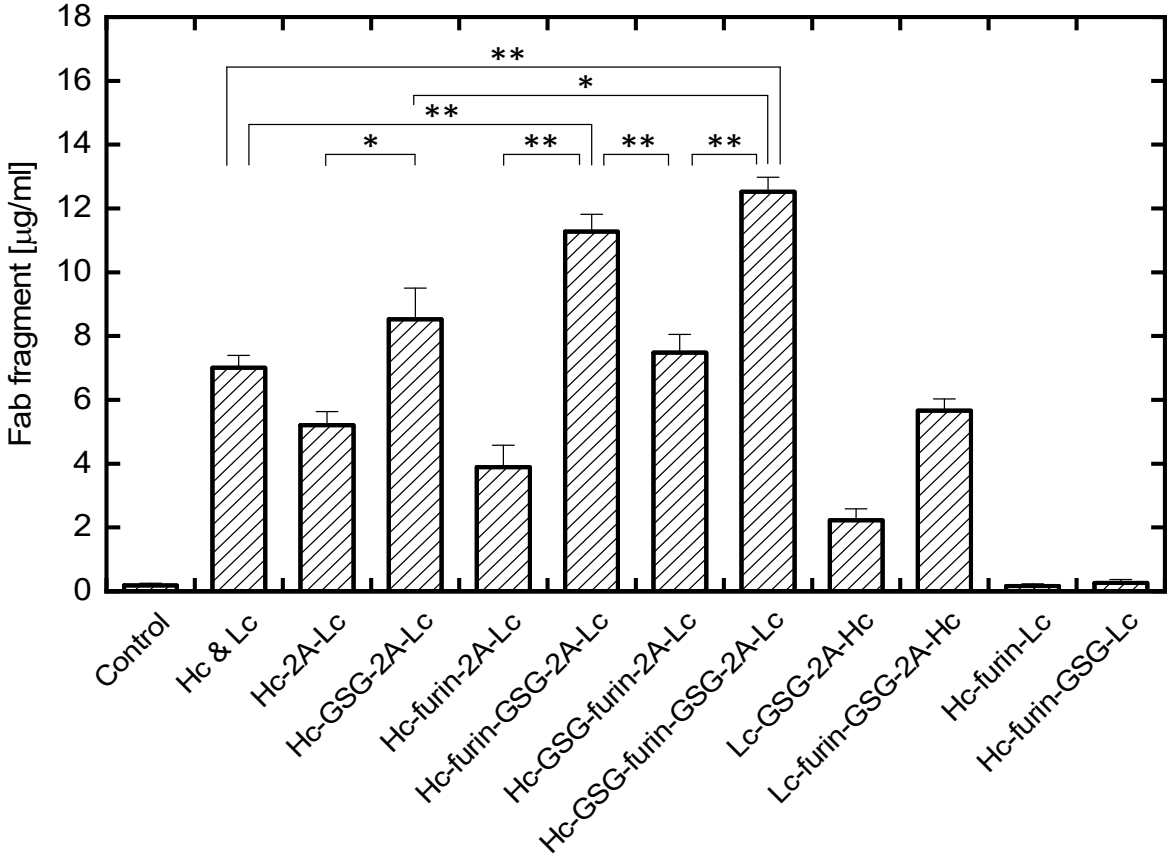
Fab fragment [μg/ml]
18
16
14
12
10
8
6
4
2
0
**
*
**
*
**
**
**
Control
Hc & Lc
Hc-2A-Lc
Hc-GSG-2A-Lc
Hc-furin-2A-Lc
Hc-furin-GSG-2A-Lc
Hc-GSG-furin-2A-Lc
Hc-GSG-furin-GSG-2A-Lc
Lc-GSG-2A-Hc
Lc-furin-GSG-2A-Hc
Hc-furin-Lc
Hc-furin-GSG-Lc

Fig. 3

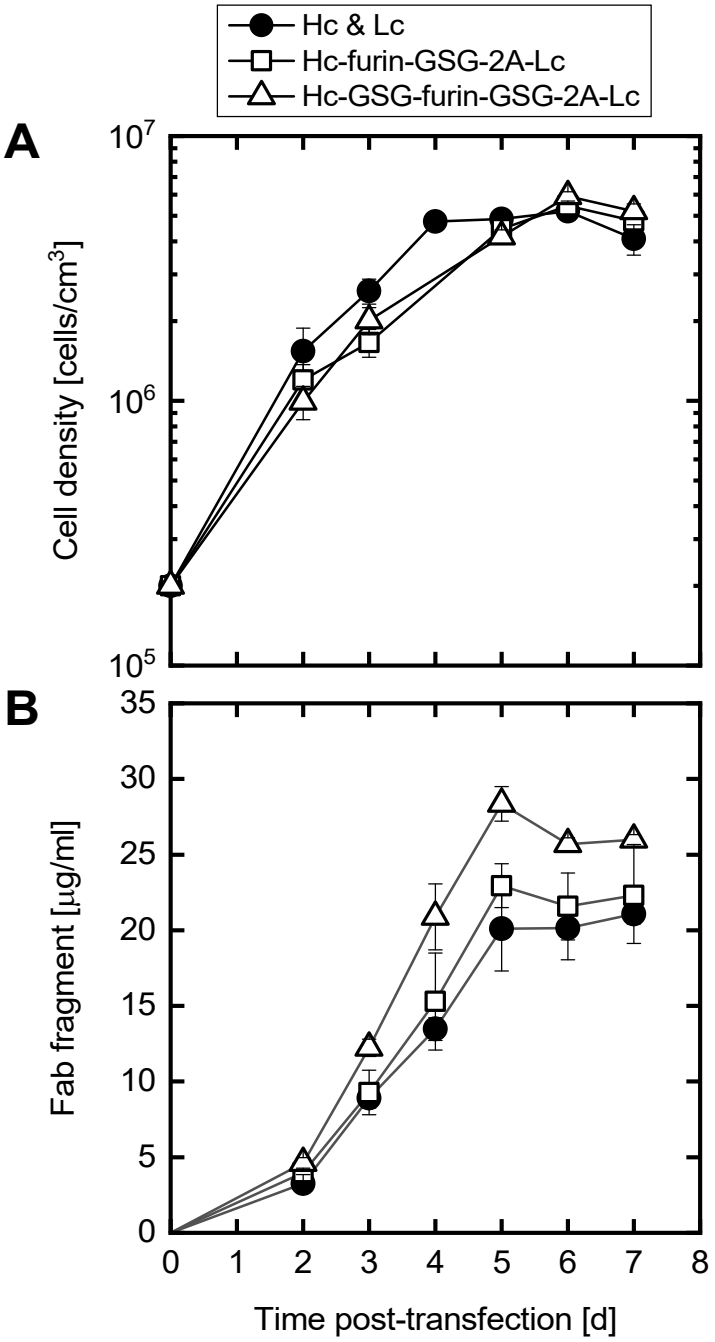
Hc & Lc
Hc-furin-GSG-2A-Lc
Hc-GSG-furin-GSG-2A-Lc
A
B
Cell density [cells/cm3]
Fab fragment [µg/ml]
Time post-transfection [d]

Fig. 4